Journal of Fish Diseases 2006, **29,** 215–224

Quantitative expression (Walbaum) of immunological factors in rainbow trout, *Oncorhynchus mykiss* (Walbaum), after infection with either *Flavobacterium psychrophilum*, *Aeromonas salmonicida*, or infectious haematopoietic necrosis virus

K Overturf[1] and S LaPatra[2]

1 USDA/ARS, Hagerman Fish Culture Experiment Station, Hagerman, ID, USA
2 Clear Springs Foods, Inc., Research Division, Buhl, ID, USA

Abstract

To further enhance our understanding of immunological gene expression in rainbow trout, *Oncorhynchus mykiss*, after infection with naturally occurring pathogens, a series of probes and primers were developed for the quantification of immune factors. Separate groups of specific-pathogen-free rainbow trout were infected with either *Flavobacterium psychrophilum*, *Aeromonas salmonicida* or infectious haematopoietic necrosis virus (IHNV). Three different concentrations of each pathogen were used and samples from infected and mock-infected fish were taken at either 1 or 5 days after infection. Ten fish were sampled at each time point for individual sections of liver, spleen and head kidney. Organ specimens from five of the fish were used to re-isolate and quantify the pathogen at the time the samples were taken. Total RNA was extracted from the organs of the remaining five animals. Using real-time polymerase chain reaction with fluorescent-labelled probes, the RNA from these organs was examined for the level of expression of the following immunological factors; an interferon related protein (MX-1), interleukin-8 (IL-8), the cytotoxic T-cell marker CD-8 and complement factor C3 (C3). They were also measured for the level of β-actin, which was used as a standardization control for cellular RNA expression. Infection with IHNV produced the greatest change in expression level for all the immunological related factors examined in this study. IHNV elicited the best dose response profile, which was typically seen at 5-days post-infection for MX-1, C3, IL-8 and CD-8. Infection with *A. salmonicida* and *F. psychrophilum* showed elevated, but variable expression levels for several of the genes tested.

Keywords: bacteria, immunological factors, quantitative expression, rainbow trout, real-time polymerase chain reaction, virus.

Introduction

With the purported increase of aquaculture in the near future and dwindling resources for rearing fish, a greater focus has been placed on increased culture intensity for aquaculture production. Attention to good husbandry and environmental conditions are of paramount concern during the intensive culture of fish. In many instances, pathogen(s) may be present, but impact of disease can be minimized if culture conditions are optimal.

The immune system of fish has been intensively studied in order to offer researchers and aquaculturists an understanding of how to optimize the environment and/or diet for enhanced fish performance and survival. Teleost fish in general have a sophisticated immune system that in some ways is comparable with mammals and higher vertebrates (Zapata, Diez, Cejalvo, Gutierrez & Cortes 2006). Their first line of defence against pathogenic agents

Correspondence *Dr K Overturf, USDA/ARS, Hagerman Fish Culture Experiment Station, 3059-F National Fish Hatchery Road, Hagerman, ID 83332, USA (e-mail: kennetho@uidaho.edu)*

© 2006 Blackwell Publishing Ltd
No claim to original US government works

includes mucus, scales and skin, which cover the body of fish and act to block attachment, penetration and infection. Internally, teleost fish combat infectious agents via their innate and specific immune systems. Innate immunity is effective at protecting the animal against a broad range of organisms and includes interferon production, activation of the complement system, the inflammatory response and the mononuclear phagocytic system (Alexander & Ingram 1992; Magnadottir 2006). The specific immune system of teleosts is relatively well-developed and able to produce both circulating antibodies and a cell-mediated response to foreign epitopes.

Researchers have isolated homologous mammalian genes and proteins of known immunological function and characterized their response in fish exposed to infectious organisms (Pleguezuelos, Zou, Cunningham & Secombes 2000; Zou, Holland, Pleguezuelos, Cunningham & Secombes 2000; Purcell, Kurath, Garver, Herwig & Winton 2004). Scientists began examining for the expression or presence of these cloned immunological factors utilizing Northern and Western blots and quantitative polymerase chain reaction (PCR) upon pathogenic agent exposure or in response to some proposed stressful or immunological compromising factor (Arkoosh & Kaattari 1991; Burrells, Williams, Southgate & Crampton 1999; Estepa, Alvarez, Ezquerra & Coll 1999; Yada & Azuma 2002). However, real-time quantitative PCR has been demonstrated as an extremely sensitive method for the analysis of immune gene activity in aquaculture research (Overturf, LaPatra & Powell 2001; Bobe, Maugars, Nguyen, Rime & Jalabert 2003; Chauvigne, Gabillard, Weil & Rescan 2003; Purcell *et al.* 2004). Utilizing this technique, molecular probes and primer sets were designed for real-time PCR to quantify the specific expression of components involved with distinct areas of the immune system. The experimental study reported here examined the expression of specific immune function genes at days 1 and 5 post-infection in fish that had been infected with one of three different fish pathogens. To account for differences noted in the past for disease resistance between viral and bacterial pathogens, two of the pathogens used in the experiment were bacterial and one was viral. The pathogens chosen for this study were *Aeromonas salmonicida*, *Flavobacterium psychrophilum* and infectious haematopoietic necrosis virus (IHNV), and the expression of interleukin-8 (IL-8), CD-8, complement factor C3, and MX-1 was assessed. Utilizing three doses of each pathogen and examining at two time points post-infection the levels of expression of these factors allows for an expanded range of coverage concerning how the animal might regulate its early defences upon invasion by intrusive pathogens. IL-8 is a cytokine involved in inflammation and attracts and activates neutrophils and T-lymphocytes. It is released by several cell types including monocytes, macrophages, T-lymphocytes, fibroblasts, endothelial cells and keratinocytes induced by an inflammatory stimulus (Stadynk 1994). CD-8 is a glycoprotein expressed on T cells that have a cytotoxic phenotype and are MHC class I restricted. CD-8 is also detected on natural killer (NK) cells in mammals, most thymocytes and bone marrow cells (Ellmeier, Sunshine, Losos & Littman 1998; Hostert, Garefalaki, Mavria, Tolaini, Roderick, Norton, Mee, Tybulewicz, Coles & Kioussis 1998; Ellmeier, Sawada & Littman 1999). Complement factor C3 is the central component of all complement reactions, split by its convertase into a small (C3a) and a large (C3b) fragment. The small soluble subunit leads to increased vascular permeability and recruitment and activation of phagocytes. C3b is deposited on the membrane where it serves as an attachment site for phagocytic polymorphs and macrophages. It acts by tagging bacteria and enhancing their recognition and hence phagocytosis by polymorphonuclear cells and macrophages (Roed, Fjalestad, Larsen & Midthjel 1992; Sunyer, Zarkadis, Sahu & Lambris 1996; Ellis 1999). MX-1 acts with interferons, which provide vertebrates with a powerful tool to defend themselves against infecting viruses. Interferons mediate their antiviral activity by inducing the synthesis of about 50 proteins. A few of the interferon-induced proteins such as MX proteins function as intracellular mediators of virus resistance. MX proteins are GTP-binding proteins and have intrinsic GTPase activity (Marlowe, Caipang, Hirono & Aoki 2003; Purcell *et al.* 2004; Acosta, Petrie, Lockhart, Lorenzen & Ellis 2005; Robertsen 2006). These four immune factors are believed to be crucial for the immunomodulatory regulation used by teleosts in dealing with disease. To gain a better understanding of how the immune system responds during early stages to low and high level bacterial and viral infections, and to determine whether gene expression could be correlated with pathogen presence, the expression level of the prior

© 2006
Blackwell Publishing Ltd
No claim to original
US government works

mentioned immune factors was quantified, after exposure to either *F. psychrophilum*, *A. salmonicida* or IHNV.

Materials and methods

Fish and rearing conditions

Specific-pathogen-free (SPF) rainbow trout, *Oncorhychus mykiss* (Walbaum), (mean weight, 14 g) were supplied by Clear Springs Foods, Inc. (Buhl, ID, USA). Fish were held in an indoor aquaculture facility and were maintained in 378-L tanks supplied with SPF 15 °C spring water treated with ultraviolet light. During the pathogen infection phase of these studies, the fish were transferred to 19-L tanks used for challenge studies. Fish were maintained by feeding an expanded trout feed (Clear Springs Foods) at 1% body weight per day or *ad libitum* after pathogen infection.

Tissue sampling and RNA isolation

At 1- and 5-days post-infection five fish were sampled for pathogen detection in the kidney, spleen and liver. Samples were processed using standard methods (Ganzhorn & LaPatra 1994). These tissues were also isolated from five additional fish from each treatment and were processed for quantitative PCR as previously described (Overturf *et al.* 2001) together with five mock infected control fish. Briefly, tissues were quick frozen in liquid nitrogen in 2-mL cryo-vials. Tissue samples were transported to the Hagerman Fish Culture Experiment Station on dry ice where they were kept at −80 °C. Within a week, the tissue samples were thawed and total RNA was isolated using the TRIZol extraction method (GIBCO, Lifetechnologies, Grand Island, NY, USA). The quantity and purity of the RNA was determined by analysis on a spectrophotometer at 260 and 280 nm and with a fluorometer using Ribogreen (Molecular Probes, Eugene, OR, USA).

Bacterial pathogen infection

A virulent strain of *F. psychrophilum* (CSF-259-93) that has been previously described (LaFrentz, LaPatra, Jones & Cain 2003) was used. The isolate was cultured in tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride and 0.05% magnesium sulphate, pH 7.2) broth and maintained at 15 °C. For challenge trials, *F. psychrophilum* was grown on TYES agar at 15 °C for 72 h. Bacteria were harvested by gentle swabbing with a cotton applicator stick and re-suspended in 0.85% saline to an optical density (OD) of 0.2, 0.4 and 0.6 at 525 nm. Twenty rainbow trout per treatment were inoculated subcutaneously with 25 μL of each OD suspension, or 0.85% saline alone, as a negative control. Plate counts were done on TYES agar at the time of subcutaneous injection of the bacteria.

A virulent strain of *A. salmonicida* (CSF-206-85) was also tested in rainbow trout. Stock 1% (w/v) bacterial suspensions were prepared in 0.5% (w/v) tryptone after growth on TS agar plates. Twenty rainbow trout were inoculated intraperitoneally with 100 μL of 1:10, 1:100 and 1:1000 dilutions of the 1% stocks, or tryptone alone as a negative control. Plate counts were done on TS agar at the time of injection of the bacteria.

Virus infection

A virulent strain of IHNV (CSF-220-90) was propagated in EPC cells using standard methods (LaPatra, Lauda & Jones 1994). Twenty rainbow trout were inoculated intraperitoneally with 100 μL of 1:10 000, 1:100 000 and 1:1 000 000 dilutions of the stock virus and 20 fish were also injected with 100 μL of saline as a control. Original stock virus concentration was 1×10^8 pfu mL^{-1}, therefore the injected concentrations were; for high dose – 1000 pfu mL^{-1}, medium dose – 100 pfu mL^{-1} and low dose – 10 pfu mL^{-1}. Virus concentrations inoculated or detected in rainbow trout tissues after infection were determined by plaque assay as previously described (LaPatra *et al.* 1994).

In vitro transcription of control mRNAs

The following sequences were used to generate control standards as previously reported (Overturf, Bullock, LaPatra & Hardy 2004). The PCR primers used for amplification of control standards were as follows: IL-8 (accession no. AJ279069) forward primer 161F 5′-GGATCC GTAACCCC AAGATGAGCATC and reverse primer 335R 5′-GAATTC GGGAACATCTCCACCTTCTTA; CD-8 alpha (accession no. AF178054) forward primer 413F 5′-GGATCC CAGCCCCTATGAC AACAACA and reverse primer 524R 5′-GAATTC GCAGTAGGGTCCACCTTTCC; C3 (accession

© 2006 Blackwell Publishing Ltd No claim to original US government works

no. L24433) forward primer 2022F 5′-GGATCC TCTGTGTCCAAGCCTTCCTT and reverse primer 2156R 5′-GAATTC CAATGTCTTCAGAC CGCATG; MX-1 (accession no. U30253) forward primer 964F 5′-TGAATTC AGCACACTTTAT GATGAGGGC and reverse primer 1160R 5′-TGGATCC AGGAAATACAGCCTCTCTGC; and the control β-actin (accession no. AF254414) forward primer 353F 5′-GGATCC TGTTGTCC CTGTACGCCTCT and 500R 5′-GAATTC TA GTCTGTGAGGTCGCGGCC.

Sequence detection

After quantification, 75 ng of total isolated RNA from each sample was added to a microcentrifuge tube containing the following; 1X TaqMan buffer, 3 mM MnOAc, 0.3 mM dNTPs except dTTP, 0.6 mM dUTP, 0.3 μM forward primer, 0.3 μM reverse primer, 0.2 μM FAM-6 (6-carboxyfluorescein) labelled probe, 5 U *rTH* DNA polymerase and 0.5 U AmpErase UNG enzyme (PE Biosystems, Foster City, CA, USA). The primers and fluorescent-labelled probes used for the mRNA detection were as follows: IL-8 forward primer – 189F 5′-AG CCAGCCTTGTCGTTGTG, reverse primer – 311R 5′-AGTTTACCA ATTCGTCTGCTTTCC, and the probe – 209T 5′-TCCTGGCCCTCCT GACCATTACTGAG; CD-8 forward primer – 445F 5′- ACACCAATGACCACAACCATAGAG, reverse primer – 518R 5′-GGGTCCACCTTTCC CACTTT, and the probe – 472T 5′-ACCAGCTC TACAACTGCCAAGTCGTGC; C3 forward primer 2060F 5′-GCCTCCAAGAAGATAGAATCC AAA, reverse primer 2143R 5′-CCGCATGTAC GCATCATCA and the probe 2085T 5′-AGG ATGCACTGCTGCTCTCACGCA; MX-1 forward primer 1044F 5′-GAAATCCCTGCCTC GTCTAGAA, reverse primer 1127R 5′-GGTCC-GGTACCATATCTTTCCA and the probe 1079T 5′-CAAAGCTGTCAGAGACACATGCCGAGC AAAGCTGTCAGAGACACATGCCGAGC; and the control probe and primer set β-actin was forward primer 372F 5′-TGGCCGTACCACCGGTAT, reverse primer 451R 5′-GCAGAGCGTAGTCCT CGTAGATG and the probe 399T 5′-CTCCGGT GACGGCGTGACCC. All probes were labelled with the fluorescent tag 6-FAM. Fifty microlitres of each reaction was pipetted into individual wells of a 96-well optical plate, capped and placed into an ABI-Prism model 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). To ensure consistency of reagent handling between each reaction, all liquid manipulation was done on a Qiagen 8000 liquid handling robot (Valencia, CA, USA). A serial dilution of six duplicate standards was run with each probe for absolute quantification. Reverse transcription and PCR conditions were as follows; 2 min at 50 °C, 30 min at 60 °C, 5 min at 95 °C and then 40 cycles of PCR consisting of 20 s at 92 °C followed by 1 min at 62 °C. The fluorescence output for each cycle of the polymerase reaction was measured and downloaded to a Macintosh G3 computer upon the completion of the entire run. Accumulated data were analysed using the computer program Sequence Detector version 1.7 (Applied Biosystems).

Data analysis

Data are reported as fold change of absolute mRNA copy number of each immune gene in infected tissue vs. control tissue standardized to the absolute mRNA copy number of β-actin. Statistical significance was calculated for the standardized expression levels between experimental groups. Excel was used to produce graphical representations of the data. Data were transformed as necessary and analysed using Student's *t*-tests and statistical correlation using Sigma Stat 2.0 (Jandel Scientific, San Rafael, CA, USA). A significance level of $P < 0.05$ was used and mean expression values were considered units of observation for statistical analysis.

Results

Detection of pathogenic organisms in the tissue of infected fish

To ensure that the changes in expression detected were linked to the presence of pathogen, and for examination of correlations of dosage level and presence of pathogen in the organ, tissues were isolated and pathogens were cultured in tissue culture. For the bacterial pathogens *A. salmonicida* and *F. psychrophilum*, the presence or absence of pathogen was monitored in the tissues (Table 1). *Aeromonas salmonicida* was detected in all tissues at both 1- and 5-days post-infection. It was also found in all fish except for the kidney of one fish at 1-day post-infection receiving the lowest dose and in the kidney of one fish that received the highest dose. At 5-days post-infection, the bacterium was found in all fish and tissues except in the kidney of one fish

© 2006 Blackwell Publishing Ltd No claim to original US government works

Table 1 Detection of *Aeromonas salmonicida* and *Flavobacterium psychrophilum* in kidney (K), spleen (S) or liver (L) of rainbow trout at 1- and 5-days post-infection

	Controls			*A. salmonicida*									*F. psychrophilum*								
	Saline/ tryptone			1.5×10^5 cfu			1.5×10^7 cfu			1.5×10^7 cfu			1.5×10^3 cfu			1.6×10^4 cfu			7.9×10^5 cfu		
Days post-infection	K	S	L	K	S	L	K	S	L	K	S	L	K	S	L	K	S	L	K	S	L
1	0/5	0/5	0/5	4/5	4/5	4/5	5/5	5/5	5/5	4/5	5/5	5/5	0/5	0/5	1/5	2/5	0/5	0/5	1/5	1/5	0/5
5	0/5	0/5	0/5	3/4	4/4	4/4	2/2	2/2	2/2	2/2	2/2	2/2	3/4	1/4	3/4	4/5	3/5	3/5	5/5	4/5	4/5

that had received the lowest dose. It should be noted that at 5-days post-infection with *A. salmonicida* three of the five fish had died so only two individuals were examined for the presence of the bacteria at these later time points. At 1-day post-infection, *F. psychrophilum* was not detected in the kidney or spleen of any fish receiving the lowest dose and only in the kidney of two fish receiving the medium injected dose. At the highest dose level of *F. psychrophilum* at 1-day post-infection, the bacterium was only detected in the kidney and spleen of one fish. However, at 5-days post-infection the presence of *F. psychrophilum* was determined in all tissues. One of the fish that died had received the lowest dose yet even at this level the bacterium was detected in the kidney and liver in three of four of the fish examined, but in the spleen of only one of these four fish. At the medium and high dose levels of *F. psychrophilum* at 5-days post-infection, the bacterium was detected in all the tissues of the majority of fish examined.

For IHNV, plaque analysis was utilized to monitor viral levels in each tissue for each time point. Although all tissues at 1-day post-infection demonstrated an increase in detected virus levels with increasing dose, interestingly, only liver showed a correlative increase for the presence of virus with increased dosage at 5-days post-infection (Fig. 1).

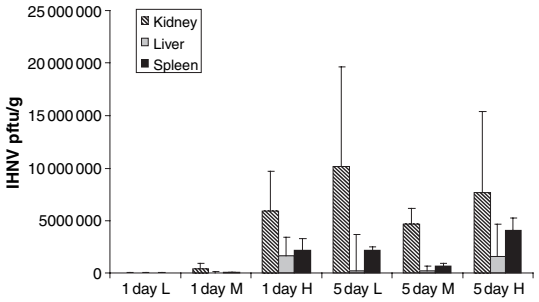

Figure 1 Detection of infectious haematopoietic necrosis virus (IHNV) in the tissues of infected fish at 1 and 5 days after infection. The dose levels were as follows; high – 1000 pfu per fish, medium – 100 pfu per fish, and low – 10 pfu per fish (L, lowest dose; M, medium dose and H, highest dose).

Comparison of immunological expression in the liver, kidney and spleen of fish at 1- and 5-days post-infection

Expression of CD-8 was significantly elevated in the spleen of IHNV infected fish at 1-day post-infection, but not at 5 days at the highest dose level (data for all tissues and factors are given in Table 2). In the liver of these animals, expression levels increased significantly at 5-days post-infection for all doses. In the kidney of IHNV infected fish, significantly elevated expression was seen at 1-day post-infection, but only in animals receiving the highest dose. For fish infected with *A. salmonicida*, a significant increase in the expression of CD-8 was detected at 5-days post-infection in the kidney at the lowest dose. In *F. psychrophilum* infected fish, CD-8 expression displayed an increase at 5-days post-infection but only in the kidney and spleen of fish infected with the medium dose.

For the immune factor IL-8, significantly elevated levels were seen at 1-day post-infection for fish infected with IHNV at a moderate dose in the liver. In the kidney and spleen at 1-day post-infection, fish receiving moderate and high doses showed a significant increase in expression, while at 5-days post-infection significant increased expression was detected in the liver and kidney only at the lowest dose. For *A. salmonicida*, increased levels were detectable at 1-day post-infection in the spleen from fish infected with the highest dose and in the liver at the medium dose. At 5-days post-infection, IL-8 was found to be increased in the kidney in animals infected at the lowest dose level. For *F. psychrophilum*, significant elevated expression was found in both spleen and kidney at 5-days post-infection at the moderate dose.

© 2006
Blackwell Publishing Ltd
No claim to original
US government works

Table 2 Fold change in expression of immune factors CD-8, complement factor C3, MX-1 and interleukin-8 in the liver, spleen and kidney of infected rainbow trout at 1- and 5-days post-infection

	Days post-infection	Liver			Kidney			Spleen		
		Low	Medium	High	Low	Medium	High	Low	Medium	High
CD-8										
Aeromonas salmonicida	1	1.32	1.07	0.21	0.32	2.92	2.71	0.13	0.57	0.91
	5	0.27	0.73	NA	0.41*	0.28	NA	0.59	0.15	NA
Flavobacterium psychrophilum	1	1.57	8.02	7.23	3.05	1.36	3.52	3.01	2.62	0.17
	5	0.28	0.88	0.32	0.30	2.24*	0.28	0.58	3.69*	0.69
Infectious haematopoietic necrosis virus (IHNV)	1	0.32	0.29	0.10	3.40	1.26	5.48*	5.75	60.33	105.99*
	5	10.7*	29.2*	56.56*	1.48	1.46	0.16	1.32	1.57	0.13
C3										
A. salmonicida	1	0.29	0.81	6.8*	0.36	1.91	2.02	1.78	1.80	1.60
	5	0.22*	0.27*	NA	17.28*	6.63	NA	0.81*	1.06	NA
F. psychrophilum	1	20.80	3.74	15.94	2.91	2.85	1.98	1.29	2.41	0.63
	5	0.20	8.95	2.40*	0.68	31.24*	8.56	6.52	6.05*	3.55
IHNV	1	8.91*	19.25*	18.8*	10.70	9.13	3.64*	0.04	1.38	1.37
	5	8.20	12.12	15.06	25.64*	6.47*	6.54	15.08*	15.53*	42.17*
MX-1										
A. salmonicida	1	0.93	50.40	4.23	0.83	22.49	1.16	617.61	16.39	157.64
	5	0.48	0.22	NA	2.13	1.16	NA	1.11	0.44	NA
F. psychrophilum	1	0.69	0.64	3.20	1.18	2.49	1.70	0.57	0.44	0.58
	5	0.47	9.53	0.68	0.14*	12.19*	1.81	0.90	9.92*	1.90
IHNV	1	183.16*	110*	17.74	0.45	92.42*	25.7*	0.34	13.01*	4.51*
	5	106.25*	576.51*	1725.39*	31.45*	222.58*	44.68	104.1*	58.69*	6.62
IL-8										
A. salmonicida	1	0.78	2.03*	19.93	0.62	1.65	4.91	0.95	2.76	16.52*
	5	0.34	0.81	NA	0.66*	0.58	NA	0.27	2.04	NA
F. psychrophilum	1	14.17	1.12	3.72	1.86	0.54	1.09	0.84	0.71	0.15
	5	0.86	2.84	0.56	0.64	3.78*	0.56	0.61	6.24*	0.59
IHNV	1	8.19	230.09*	4.14	0.96	5.46*	5.85*	0.60	64.56*	37.29*
	5	2.77*	3.26	2.20	7.0*	1.55	0.20	2.85	3.22	0.55

*Indicates fold change is significant $P < 0.05$.

For complement factor C3, IHNV infected fish showed significant differences for increased expression levels in all tissues. In the liver, increased levels were found at all dose levels at 1-day post-infection. In the kidney, these changes were detected at low and medium doses at 5-days post-infection, and at the highest dose at 1-day post-infection. In the spleen, the levels of change in expression were significant at 5-days post-infection at all doses of virus. With *A. salmonicida*, elevated levels were detectable in the liver at 1-day post-infection with a high dose of pathogen and for all doses after 5 days. In the spleen and the kidney, significant differences were only detected for the low dose at 5-days post-infection. *Flavobacterium psychrophilum* showed significant increases in the level of C3 expression at 5-days post-infection in the spleen and kidney with a medium dose of the pathogen, and in the liver only at day 5 post-infection at the highest dose level.

Expression of MX-1 in the liver of IHNV infected fish was significantly elevated for all doses and at all time points except at 1-day post-infection at the highest dose. In the kidney, expression was significantly elevated at 1-day post-infection with a high dose of pathogen and at the low and medium doses at 5-days post-infection. In the spleen, MX-1 expression was significantly elevated at all times and doses except at 1-day post-infection at the lowest dose and day 5 post-infection at the highest dose. With *A. salmonicida*, there were no significant expression changes found in any of the tissues at either time point. For *F. psychrophilum* MX-1 expression was found to be significantly elevated in the spleen at day 5 post-infection at the medium dose of the pathogen. In kidney, significant differences were detected at 5-days post-infection in fish injected with either the low or medium dose of the pathogen. No significant expression differences were found in the liver.

Correlation of immunological expression with pathogen dose

In most instances, a change in dose did not correlate either in a positive or negative manner with a

© 2006
Blackwell Publishing Ltd
No claim to original
US government works

change in expression level. Correlative changes detected in this study were most often seen with IHNV. In the liver, at 5-days post-infection, IHNV demonstrated a positive correlation of elevated expression with increased pathogen dose for CD-8, MX-1 and C3 with correlation coefficients of 0.99, 0.94 and 0.97, respectively. However, it must be noted that for C3, the expression changes were not considered significantly different from the control. Also, at 1-day post-infection in the liver there was a significant negative correlation for the expression of MX-1 with viral dose (−0.93), but the change in expression at the highest dose was again not significant from the control. In the spleen, IHNV demonstrated positive correlations with CD-8 at 1-day post-infection (0.88), although the low and medium doses were significantly different, and for C3 at 5-days post-infection (0.99).

For *A. salmonicida*, correlative changes were detected in the liver for CD-8 (−0.99) and IL-8 (0.99), however, IL-8 was significantly differentially expressed at the medium dose. The same factors were also correlatively expressed with the pathogen at 1-day post-infection in the spleen (0.87 and 0.99, respectively), however, for IL-8 the differences were significant only at the high dose.

For *F. psychrophilum*, correlative changes were only found at 1-day post-infection and they were all negative. For CD-8 and IL-8, these changes were found in the spleen (−0.99 and −0.95, respectively) and for C3 they were found in the kidney (−0.97). Though the changes were correlative, none of these original measurements had been determined to be significantly different from the controls at a value of $P < 0.5$.

Discussion

In the results presented in this report, IHNV appeared to generate the most significant immune response. At 5-days post-infection, significant increases in the expression of CD-8 and MX-1 were seen in the liver for which increasing doses of virus highly correlated with increased gene expression. Interestingly, at 1-day post-infection in the liver and 5-days post-infection in the spleen, IHNV dose demonstrated a negative correlation with MX-1 gene expression, although expression levels from the highest dose were not significant at $P < 0.5$. Other groups who have examined MX-1 expression after immunological induction have typically seen an increase in the level of MX-1 after 1 day, which continued until reaching a peak between 3 days and 2 weeks (Lockhart, Bowden & Ellis 2004; Purcell *et al.* 2004; Acosta *et al.* 2005). One research group analysed MX-1 expression in Atlantic salmon with two different doses of a bacterium (*Listonella anguillarum*), lipopolysaccharide and chromosomal DNA and they also saw an increase in expression with increasing levels of pathogen only in the liver (Acosta, Lockhart, Gahlawat, Real & Ellis 2004). In contrast to these previous studies, few significant changes in expression were found for MX-1 expression after infection with either *A. salmonicida* or *F. psychrophilum*.

Expression changes in CD-8 were also found to be significant and correlative with IHNV dose in the liver at 5-days post-infection and correlative in the spleen at 1-day post-infection. CD-8 is primarily known as a marker for cytotoxic T-cells, but it is also expressed on a number of other immune cells such as NK cells in mammals which are considered to be the equivalent non-specific cytotoxic cells in fish (Greenlee, Brown & Ristow 1991; Evans & Jaso-Friedman 1992). In mammals, NK cells are mainly found in the liver and spleen, whereas in fish these cells have mostly been examined for in the spleen and anterior kidney (Evans, Carlson, Graves & Hogan 1984; Greenlee *et al.* 1991; Abbas, Lichtman & Pober 1994). In comparison with other published studies we found relatively few significant changes occurring in the kidney of infected fish, while significant and correlative increases in expression occurred in the liver and the spleen with IHNV and similar changes were also found after infection with bacterial pathogens. The kidney is a major organ for the capture and clearance of bacteria because of the significant populations of macrophage and endothelial cells that reside there (Ferguson, Claxton, Moccia & Wilkie 1982; Kaattari & Irwin 1985; Press & Evensen 1999). However, from the results of this study it could be speculated that most of the lymphocytic action, or recruitment and activation of these cells, that occurs after infection is resolved in the liver and the spleen.

Rainbow trout have a complement system that is analogous to mammals and plays an important role in defence against infection (Tomlinson, Stanley & Esser 1993; Sunyer *et al.* 1996). The expression of complement factor C3 determined in this study followed the general pattern detected for CD-8 and MX-1, in that correlative changes were seen in the liver at 1-day post-infection and correlative and

© 2006
Blackwell Publishing Ltd
No claim to original
US government works

significant differences were detected in the liver at 5-days post-infection. Correlative, but non-significant changes were detected in the kidney and spleen for fish infected with *F. psychrophilum*, but not with *A. salmonicida*. As with MX-1, it was found that IHNV generates the strongest immune response for the activation of this pathway. A study examining expression of immune genes after exposure to the parasitic ciliate *Ichthyophthirius multifiliis* found C3 transcription induced in the skin and the spleen and deduced an enhanced expression from the liver (Sigh, Lindenstrom & Buchmann 2004a). These authors found that significant increases were not detected until after 4 days and peaked at 6 days which is in agreement with the present study for the liver and spleen. They also observed a depression in the expression of C3 in the head kidney, as was detected in this study with fish infected with all three pathogens.

In mammals, IL-8 functions as a chemokine for the attraction of leucocytes to an area of infection. It was one of the first interleukin sequences identified in fish and it has been presumed to play the same role in teleost fish as it does in mammals (Sangrador-Vegas, Lennington & Smith 2002). In this study, correlative changes were detected in all tissues but not for all pathogens. *Aeromonas salmonicida* demonstrated a correlative increase in the expression of IL-8 at 1-day post-infection in the liver. Bridle, Morrison & Nowak (2006) found a significant increase in the expression of IL-8 in the liver of trout infected with amoebic gill disease, but no significant differences were determined at any time prior to or after 7 days. IHNV infection showed the same type of response in kidney with the two higher doses being significantly increased. Sigh, Lindenstrom & Buchmann (2004b) found a significant increase in the expression of IL-8 in head kidney only after 26 days in trout infected with *I. multifiliis* but not at earlier time points, while Bridle *et al.* (2006) found no significant changes in IL-8 expression. We found a significant correlative increase of dose with expression of IL-8 in the spleen of animals infected with *A. salmonicida* at the highest dose. In trout infected with *F. psychrophilum*, there was a relative decrease in the expression of IL-8 with increasing dose, but none of these differences were significant. In other studies examining IL-8 expression in the spleen, Sigh *et al.* (2004b) detected significant upregulation at days 4, 6 and 26 after infection with *I. multifiliis*, while Purcell *et al.* (2004) detected significant expression in the spleen of fish infected with IHNV at days 3 and 7 post-infection, but not after 1 day. In this study we did detect significant, but not correlative, increases in the liver of fish infected with IHNV, but only at 1-day post-infection.

In summary, from the findings reported herein it would appear that each pathogen, whether viral or bacterial, was dealt with in a distinctive manner by the immune system of rainbow trout. This is perhaps not surprising considering the variations in the pathogenesis for each of the infectious organisms used in the study. From the greatly enhanced fold change expression for some genes at a specific dose of pathogen and time post-infection, it would appear that there are optimal infection methods for stimulating specific components of the immune system. Research evaluating the immune system in humans acknowledges the inherent problems in analysing the immune response and variations between individuals (Cummings, Antoine, Azpiroz, Bourdet-Sicard, Brandtzaeg, Calder, Gibson, Guarner, Isolauri, Pannemans, Shortt, Tuijtelaars & Watzl 2004; Fierz 2004). Although it is tempting to suggest what might be occurring physiologically within the animal, these results only demonstrate a correlation, where present, between gene expression and a known infection of a pathogen. Further analysis of protein levels, immunological assays and ligand-binding studies will need to be undertaken to more fully understand the immunological response that is occurring. However, these analyses do provide a starting point for monitoring and evaluating immunological responses that occur during pathogen infection in rainbow trout.

Acknowledgements

The authors would like to acknowledge Jerry Jones, Bill Shewmaker and Aaron Weighall from Clear Springs for their excellent technical assistance. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

Abbas A., Lichtman A. & Pober J. (1994) *Cellular and Molecular Immunology*, 2nd edn. W.B. Saunders Company, Philadelphia, PA.

© 2006
Blackwell Publishing Ltd
No claim to original
US government works

Acosta F., Lockhart K., Gahlawat S., Real F. & Ellis A. (2004) Mx expression in Atlantic salmon (*Salmo salar* L.) parr in response to *Listonella anguillarum* bacterin, lipopolysaccharide and chromosomal DNA. *Fish and Shellfish Immunology* **17,** 255–263.

Acosta F., Petrie A., Lockhart K., Lorenzen N. & Ellis A. (2005) Kinetics of Mx expression in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar* L.) parr in response to VHS-DNA vaccination. *Fish and Shellfish Immunology* **18,** 81–89.

Alexander J. & Ingram G. (1992) Noncellular nonspecific defence mechanisms of fish. *Annual Review of Fish Diseases* **2,** 81–92.

Arkoosh M.R. & Kaattari S.L. (1991) Development of immunological memory in rainbow trout (*Oncorhynchus mykiss*). I. An immunochemical and cellular analysis of the B cell response. *Developmental and Comparative Immunology* **15,** 279–293.

Bobe J., Maugars G., Nguyen T., Rime H. & Jalabert B. (2003) Rainbow trout follicular maturational competence acquisition is associated with an increased expression of follicle stimulating hormone receptor and insulin-like growth factor 2 messenger RNAs. *Molecular Reproductive Development* **66,** 46–53.

Bridle A., Morrison R. & Nowak B. (2006) The expression of immune-regulatory genes in rainbow trout, *Oncorhychus mykiss*, during amoebic gill disease (AGD). *Fish and Shellfish Immunology* **20,** 346–364.

Burrells C., Williams P.D., Southgate P.J. & Crampton V.O. (1999) Immunological, physiological and pathological responses of rainbow trout (*Oncorhynchus mykiss*) to increasing dietary concentrations of soybean proteins. *Veterinary Immunology and Immunopathology* **72,** 277–288.

Chauvigne F., Gabillard J.C., Weil C. & Rescan P.Y. (2003) Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. *General and Comparative Endocrinology* **132,** 209–215.

Cummings J., Antoine J., Azpiroz E., Bourdet-Sicard R., Brandtzaeg P., Calder P., Gibson G., Guarner F., Isolauri E., Pannemans D., Shortt C., Tuijtelaars S. & Watzl B. (2004) PASSCLAIM – gut health and immunity. *European Journal of Nutrition* **43,** II118–II173.

Ellis A. (1999) Immunity to bacteria in fish. *Fish and Shellfish Immunology* **9,** 291–308.

Ellmeier W., Sunshine M.J., Losos K. & Littman D. (1998) Multiple developmental stage-specific enhancers regulate CD-8 expression in developing thymocytes and in thymus-independent T cells. *Immunity* **9,** 485–496.

Ellmeier W., Sawada S. & Littman D. (1999) The regulation of CD4 and CD-8 coreceptor gene expression during T cell development. *Annual Reviews in Immunology* **17,** 523–554.

Estepa A., Alvarez F., Ezquerra A. & Coll J.M. (1999) Viral-antigen dependence and T-cell receptor expression in leucocytes from rhabdovirus immunized trout. *Veterinary Immunology and Immunopathology* **68,** 73–89.

Evans D. & Jaso-Friedman L. (1992) Nonspecific cytotoxic cells as effectors of immunity in fish. *Annual Review of Fish Disease* **2,** 109–121.

Evans D., Carlson R., Graves S. & Hogan K. (1984) Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*). IV. Target cell binding and recycling capacity. *Developmental and Comparative Immunology* **8,** 823–833.

Ferguson H., Claxton M., Moccia R. & Wilkie E. (1982) The quantitative clearance of bacteria from the bloodstream of rainbow trout (*Salmo gairdneri*). *Veterinary Pathology* **19,** 687–699.

Fierz W. (2004) Basic problems of serological laboratory diagnosis. *Methods in Molecular Medicine* **94,** 393–427.

Ganzhorn J. & LaPatra S. (1994) General procedures for viruses. In: *Suggested Procedures for the Detection and Identification of Certain Finfish and Shellfish Pathogens*, 4th edn (ed. by J.C. Thoesen), pp. 1–14. American Fisheries Society, Bethesda, MD.

Greenlee A., Brown R. & Ristow S. (1991) Nonspecific cytotoxic cells of rainbow trout (*Oncorhynchus mykiss*) kill YAC-1 targets by both necrotic and apoptic mechanisms. *Developmental and Comparative Immunology* **15,** 153–164.

Hostert A., Garefalaki A., Mavria G., Tolaini M., Roderick K., Norton T., Mee P., Tybulewicz V., Coles M. & Kioussis D. (1998) Hierarchical interactions of control elements determine CD-8α gene expression in subsets of thymocytes and peripheral T cells. *Immunity* **9,** 497–508.

Kaattari S. & Irwin M. (1985) Salmonid spleen and anterior kidney harbor population of lymphocytes with different B cell repertoires. *Developmental and Comparative Immunology* **9,** 433–444.

LaFrentz B.R., LaPatra S.E., Jones G.R. & Cain K.D. (2003) Passive immunization of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease and rainbow trout fry syndrome. *Journal of Fish Diseases* **26,** 377–384.

LaPatra S.E., Lauda K.A. & Jones G.R. (1994) Antigenic variants of infectious hematopoietic necrosis virus and implications for vaccine development. *Diseases of Aquatic Organisms* **20,** 119–126.

Lockhart K., Bowden T. & Ellis A. (2004) Poly I:C-induced Mx responses in Atlantic salmon parr, post-smolts and growers. *Fish and Shellfish Immunology* **17,** 245–254.

Magnadottir B. (2006) Innate immunity of fish (overview). *Fish and Shellfish Immunology* **20,** 137–151.

Marlowe C., Caipang A., Hirono I. & Aoki T. (2003) *In vitro* inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx. *Virology* **317,** 373–382.

Overturf K., LaPatra S.E. & Powell M. (2001) Real-time PCR for the detection and quantitative analysis of IHNV in salmonids. *Journal of Fish Diseases* **24,** 325–333.

Overturf K., Bullock D., LaPatra S. & Hardy R. (2004) Genetic selection and molecular analysis of domesticated rainbow trout for enhanced growth on alternative diet sources. *Environmental Biology of Fishes* **69,** 409–418.

Pleguezuelos O., Zou J., Cunningham C. & Secombes C.J. (2000) Cloning, sequencing, and analysis of expression of a second IL-1 beta gene in rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics* **12,** 1002–1011.

© 2006
Blackwell Publishing Ltd
No claim to original
US government works

Press C. & Evensen O. (1999) The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology* **9,** 309–318.

Purcell M., Kurath G., Garver K., Herwig R. & Winton J. (2004) Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection of DNA vaccination. *Fish and Shellfish Immunology* **17,** 447–462.

Robertsen B. (2006) The interferon system of teleost fish. *Fish and Shellfish Immunology* **20,** 172–191.

Roed K., Fjalestad K., Larsen H. & Midthjel L. (1992) Genetic variation in haemolytic activity in Atlantic salmon (*Salmo salar* L). *Journal of Fish Biology* **40,** 739–750.

Sangrador-Vegas A., Lennington J. & Smith T. (2002) Molecular cloning of an IL-8-like CXC chemokine and tissue factor in rainbow trout (*Oncorhynchus mykiss*) by use of suppression subtractive hybridization. *Cytokine* **17,** 66–70.

Sigh J., Lindenstrom T. & Buchmann K. (2004a) Expression of pro-inflammatory cytokines in rainbow trout (*Oncorhynchus mykiss*) during an infection with *Ichthyophthirius multifiliis*. *Fish and Shellfish Immunology* **17,** 75–86.

Sigh J., Lindenstrom T. & Buchmann K. (2004b) The parasitic ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **27,** 409–417.

Stadynk A. (1994) Cytokine production of epithelial cells. *FASEB* **8,** 1041–1047.

Sunyer J., Zarkadis I., Sahu A. & Lambris J. (1996) Multiple forms of complement C3 in trout that differ in binding to complement activators. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 8546–8551.

Tomlinson S., Stanley K. & Esser A. (1993) Domain structure, functional activity, and polymerization of trout complement protein C9. *Developmental and Comparative Immunology* **17,** 67–76.

Yada T. & Azuma T. (2002) Hypophysectomy depresses immune functions in rainbow trout. *Comparative Biochemistry and Physiology C* **131,** 93–100.

Zapata A., Diez B., Cejalvo T., Gutierrez C. & Cortes A. (2006) Ontogeny of the immune system of fish. *Fish and Shellfish Immunology* **20,** 126–136.

Zou J., Holland J., Pleguezuelos O., Cunningham C. & Secombes C.J. (2000) Factors influencing the expression of interleukin-1 beta in cultured rainbow trout (*Oncorhynchus mykiss*) leucocytes. *Developmental and Comparative Immunology* **6,** 575–582.

Received: 5 October 2005
Revision received: 7 February 2006
Accepted: 7 February 2006

© 2006
Blackwell Publishing Ltd
No claim to original
US government works